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RESEARCH PAPER

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Toxicity effects of AgZnO nanoparticles and rifampicin on *Mycobacterium tuberculosis* into the macrophage

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The World Health Organization acknowledges tuberculosis as a global threat. Tuberculosis infection is one of the top 10 causes of death worldwide. Nanotechnology and microbiology researchers are looking for new and safe nano drugs for eliminating *Mycobacterium tuberculosis*, the causative agent of tuberculosis. In this study, AgZnO nano-crystals (AgZnONCs) is synthesized via the decomposition of the precursor of oxalate method. Characterization of AgZnONCs were evaluated. Next, various concentrations of AgZnONCs, as well AgZnONCs+Rifampicin, were prepared. The MTT assay was employed to study the viability of human macrophage cell lines (THP-1) exposed to AgZnONCs. The bactericidal effects of AgZnONCs and AgZnONCs+Rifampicin were studied by Minimum Bactericidal Concentration (MBC) test. Subsequently, THP-1 were infected by $H_{37}Rv$ strain of *M. tuberculosis* ($H_{37}RvMtb$). Also, bactericidal effects of AgZnONCs and AgZnONCs+Rifampicin were compared with *ex-vivo* conditions. The MBC of AgZnONCs and AgZnONCs+Rifampicin were ratios of 1:4 and 1:32 respectively (p-value <0.05). Also, more than 50% and 80% of THP-1 were alive in ratios of 1:4 and 1:32 in the presence of AgZnONCs, respectively. All phagocytic $H_{37}RvMtb$ were killed in the presence of AgZnONCs+Rifampicin (p-value <0.05),

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while AgZnONCs were not able to kill all the $H_{37}RvMtb$ (p-value >0.05). This study showed that, AgZnONCs+Rifampicin has the most anti-tubercular behavior with respect to the macrophages.

KEYWORDS

AgZnO nano-crystals, bactericidal, macrophage, *Mycobacterium tuberculosis*, phagocytosis, rifampicin

1 | INTRODUCTION

According to the last World Health Organization reports, it is estimated that around 5.4 million male, 3.2 million female, and about 1 million children are suffering from tuberculosis in the world which, about 1.5 million people have lost their lives in 2014 [1]. About 3.5% of people which recently tuberculosis (TB) were diagnosed and about 20.5% of patients who were previously treated, had multidrug resistant tuberculosis (MDR-TB) in which half of those patients did not complete their prescribed drug regimen. The problem of tuberculosis patients is not only the *Mycobacterium tuberculosis* (*Mtb*) resistance to rifampicin or isoniazid but also to other anti-TB drugs. *Mtb* penetrates by enters to the respiratory tract through ultrafine infectious particles into alveoli, where by alveolar macrophages are phagocytized. *Mtb* is able to prevent its connection with phagosomes or lysosomes. The phagocytized bacteria are able to escape from nitrogen-containing active agents, oxidase and catalase agents [2]. Due to related factors to *Mycobacterial* infection, tuberculosis treatment presented issues during chemotherapy. Due to the chemotherapy, the body's defense mechanism is inefficient, and the patient is considered to be immunocompromized. Although many people are affected with TB in a latent state, only a small percentage of people actually develop active, symptomatic pulmonary TB. In addition, pathological anatomy of lesions with high leukocytes infiltration and central calcification, drugs penetration to the appropriate concentrations becomes too difficult and antibiotics usually do not have access to the *Mtb*. Most anti-TB drugs have bacteriostatic activity and requires a lengthy treatment. Other difficulties in TB treatment are the emergence of resistance in microorganisms, and finally toxic side effects of drugs hinders their use or forces the prescription of low-doses. Using the combination therapy will prevent the emergence of resistance [2,3]. Today, nanotechnology is used widely in various aspects in medical sciences and basic sciences. Researchers believed that colloidal nanoparticles and agglomerates are able to limit the growth of bacteria and even destroy their cellular structure [4]. Many studies have been conducted on the antibacterial effects of Ag and ZnO nanoparticles [5–9]. Silver has always been the most promising candidate to be investigated for its inhibitory and antibacterial properties since ancient times [10]. In fact, Ag ions and Ag-based compounds are highly toxic to microorganisms, showing biocidal effects on pathogenic bacteria [11]. On the other hand, using just silver nanoparticles may be ineffective against some bacteria, because the antibacterial properties of silver nanoparticles are weak. Based on previous studies, synergistic effects of silver and zinc oxide nanoparticles (AgZnONPs) have been proven effective on various bacteria [12–14]. Zinc oxides has been recognized as antibacterial material in recent years. For several decades, they have been used in medicine as mild topical astringents, and antibacterial agents against eczema, slight excoriations, in wounds, and for hemorrhoids [11]. Zinc oxide has been approved as a safe material by the U.S Food and Drug Administration (FDA, 2011) and its antibacterial activity has been long known [11]. Therefore, silver and zinc oxide nano-crystals (NCs) offer potential applications in antimicrobial coatings and for coating surgical instruments to prevent nosocomial infections [10]. Scientists reported antimicrobial activity of silver nanoparticles in combination with zinc oxide nanoparticles [11]. Rifampicin, a semisynthetic antibiotic for *Mycobacterium* and methicillin-resistant *Staphylococcus aureus* infection treatment [15]. Recently, researchers are interested to investigating about synergism and antibacterial effects of metal oxide nano-crystals (NCs) containing currency antibiotics such as rifampicin [15].

In this study, we explored the toxicity of silver/zinc oxide nano-crystals (AgZnONCs) on the human macrophages (THP-1) cell lines and investigated synergistic effects of mixed AgZnONCs with rifampicin, before (*in vivo*) and after (*ex vivo*) phagocytosis against $H_{37}RvMtb$.

2 | MATERIALS AND METHODS

2.1 | Characterization of NCs, chemicals, $H_{37}RvMtb$ strains, growth media, and THP-1 cell lines

Experimental studies were performed February 1, 2016 till September 14, 2016 at Institute of Immunology and Infectious Diseases (IIID), Anti-microbial Resistance Research Center, Iran University of Medical Sciences (IUMS). The AgZnONCs were synthesized and its properties were

investigated [16]. Experiences of dependent on the crystallinity of the NPs were carried out using X-ray diffract meter set (XRD, Bruker D8-Advance Diffract meter using Cu Kα radiation). Fourier transform infrared (FT-IR) spectra were recorded on a Bruker spectrophotometer in KBr pellets. Surface morphology of product was characterized by using a scanning electronic microscopy (SEM, Cam Scan MV2300) with an accelerating voltage of 30 KV. Also, different dilutions of NCs were prepared (Tables 4 and 5). Next, the concentration of NCs was determined at each dilution [10]. *H_{37}RvMtb* (ATCC 27294) was provided from Razi Vaccine and Serum Research Institute. THP-1 cell line was obtained from cell bank of Pasteur Institute of Iran and next, was cultured in RPMI 1640 medium (Thermo Fisher Scientific, USA), FBS 10%, penicillin 100 U ml^{-1} and 100 mg ml^{-1} of streptomycin (Sigma–Aldrich, USA) and then incubated at 5% CO_2 at 37 °C. When cell density reached to 80%, THP-1 cells (about 10^4 cell/well) were cultured in each plate.

2.2 | Preparation of *H_{37}RvMtb* and different dilutions of NCs

Two colonies of *H_{37}RvMtb* were removed from Lowenstein Jensen (LJ) agar medium (Fisher Bio Reagents, USA) and were transferred into gated test tube containing 5 ml of sterile saline and was stirred for 5–10 min with a shaker device. The opacity in the test tube containing the intended bacteria was compared with opacity in a 0.5 McFarland test tube, macroscopically. In order preparation of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions of *H_{37}RvMtb*, four gated test tube containing 5 ml of sterile saline were prepared and serial dilutions were done. Also, to prepare AgZnONCs+Rifampicin, 0.16384 g of AgZnONCs and 0.0008 g of rifampicin were mixed into the 20 ml of LJ agar medium.

2.3 | Anti-tubercular activity of AgZnONCs and AgZnONCs+rifampicin

To investigation about anti-tubercular activity of NCs, Minimum Bactericidal Concentration (MBC) test was used. About 0.32768 g of AgZnONCs was poured separately into the sterile tube, containing 20 ml of melted LJ agar medium as a negative control and after shaking, it was ultra-sonicated for 10 min in room temperature. After pipetting, about 10 ml of melted LJ agar containing AgZnONCs was added to the subsequent tube containing 10 ml of melted LJ agar medium. Serial dilution was done. Last tube was treated as a positive control. To MBC tests, about 10^{-2} (75×10^6 CFU ml^{-1}) and 10^{-4} (18.7×10^4 CFU ml^{-1}) dilutions of *H_{37}RvMtb* were prepared and likewise, about 100 ml of *H_{37}RvMtb* were added to each tubes and kept in an incubator at 37 °C for 28 days. Then, tubes were removed from the incubator and the colony forming units (CFU) were counted accurately for any dilution (Table 1). Meanwhile, MBC test of AgZnONCs+Rifampicin against *H_{37}RvMtb* was performed in accordance with the above procedure. Also, MBC tests were performed three times.

2.4 | MTT assay

The cytotoxicity effects of AgZnONCs on THP-1 cells in 24 h were determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. The cells ($10^4_{cell/well}$) were seeded in RPMI-1640 (Thermo Fisher Scientific), medium (200 ml per well) in 96-well plates and incubated at 37 °C in 5% CO_2 for 24 h, to induce cell adherence. Next, serial dilution was done. Separately, THP-1 cells were treated with 100 µl of freshly prepared AgZnONPs and further incubated at 37 °C in 5% CO_2 for 48 h. To the MTT assay, 20 µl of MTT solution (5 mg ml^{-1} in incomplete medium) was added and incubated for 5 h. Next, 100 µl DMSO was added and the plates put on a shaker for 5 min. The maximum absorbance was measured at 560 nm using a microtiter plate reader (Biotech, USA) [17]. Cell viability in control cells was assumed to be 100%. The percentage of cells viability were calculated by using the following equation:

$$\%\text{THP-1 cells viability} = \frac{\text{Optical Density (OD) of test}}{\text{OD of controls}} \times 100$$

2.5 | The infectious THP-1 cell lines by *H_{37}RvMtb* and treated with NCs

The infectious THP-1 cell lines with *H_{37}RvMtb* was performed under five steps. In first step, RPMI-C medium ("RPMI-Complete") was prepared (Table 2). A day before infection, 50 nM of phorbol myristate acetate (PMA) (SIGMA, USA) were added to THP-1 cell lines in RPMI-C medium. Next, RPMI-I medium ("RPMI-infection") was ready (Table 3). One day before infection, about 10^{-2} and 10^{-4} McFarland of *H_{37}RvMtb* were prepared. Also, about 100 µl of THP-1 cells (1×10^5 cells/well) were placed into 96-well micro-plate. Then, macrophages were washed with PBS and then 100 ml of RPMI-I medium culture were added and cells were incubated. After that, three ml of suspension of different dilution of *H_{37}RvMtb* were centrifuged for ten minutes at 2400 rpm, and three ml of fresh 7H9-C medium culture were added. Next step, *H_{37}RvMtb* and THP-1 cell lines were mixed in RPMI-I medium; afterwards, infected macrophages were incubated at 37 °C for 3 h under 0.5% CO_2. Macrophages were washed twice with PBS at 37 °C to remove extracellular bacteria. Then, RPMI-C containing 20 µg ml^{-1} gentamicin were added to medium. Fourth step, different ratios of AgZnONCs and AgZnONCs+Rifampicin were used to treatment of infected macrophages. Afterwards,

TABLE 1 The resistance ratio of the number of colonies observed in LJ agar medium

Symbol	Colony count of *Mtb*
+1	150–300
+2	300–500
+3	~500
+4	500≤

they were incubated at 37 °C for 24 h under 0.5% CO_2. Next, macrophages were lyzed by 0.05% SDS and cultured in LJ agar medium. About 25 µl of lyzed cells were transferred to the new micro-plate containing 225 µl of MGIT broth medium (Sigma), until the serial dilution was done. Next step, 100 µl of the selected dilution were cultured in LJ agar medium.

2.6 | Preparation to TEM and SEM

For preparing of transmission electron microscopy (TEM) (Philips CM30, USA) image of the infectious THP-1 and NCs, it was fixed by glutaraldehyde 2.5% (Sigma–Aldrich). Then, it was washed by Sodium cacodylate trihydrate (Sigma–Aldrich) buffer in two steps, one step for 15 min and another for 30 min. After washing, the suspension was fixed by osmium tetroxide 1% (Sigma–Aldrich) immediately. Next step, dehydration was performed with ethanol 50%, 70%, 90%, and 100%, respectively. The contents of the cell were immersed in pure propylene oxide (Sigma–Aldrich) and various degrees of propylene oxide and resin (Sigma–Aldrich), and then were placed in pure resin. After that, the sample was placed in oven at 60 °C for 24 h. Finally, the obtained samples were cut using ultra-microtome (Reichert-Leica, USA) in very thin pieces and painted by uranyl acetate 2% (Sigma–Aldrich) and Lead (II) citrate tribasic trihydrate 2% (Sigma–Aldrich) and were placed on the special grades by TEM. To identification and analysis of AgZnONCs, scanning electron microscope (SEM) (Hitachi S4160, Japan) was used. NCs were diluted in distilled water and poured on copper Stub. Then, NCs were coated by a thin layer of gold under vacuum conditions to be conductive. Surface covered with gold was scanned and photography was performed by accelerating voltage 10 kV.

TABLE 2 Preparation of RPMI-C medium

RPMI-C mediums		
Material	**Volume**	**Company**
RPMI media	450 ml	Thermo fisher Scientific
HEPES	1% (or 5 ml) 1 mM	Sigma–Aldrich
2-Mercaptoethanol	0.1% (or 500 µl)	Sigma–Aldrich
Gentamicin	20 µg ml^{-1}	Sigma–Aldrich

TABLE 3 Preparation of RPMI-I medium

RPMI-I medium		
Material	**Volume**	**Company**
RPMI media	450 ml	Thermo fisher Scientific
HEPES	1% (or 5 ml) 1 mM	Sigma–Aldrich
2-Mercaptoethanol	0.1% (or 500 µl)	Sigma–Aldrich
Non-heat-inactivated human serum	10%	Gemini Bio-products

2.7 | Statistical analysis

In this study, statistical data analysis was performed by SPSS 16 software. Significant difference between the different concentrations of AgZnONCs on *H_{37}RvMtb* were studied by analysis of variance one-way ANOVA and the comparison of data in alpha level of 0.05 and confidence 95%. The diagrams were drawn with Microsoft Excel (version of 2017). Experiments were performed three times.

3 | RESULTS

3.1 | Characterization of AgZnONCs

Characterizations of AgZnONCs were consistent with previous studies of Jafari et al. The FTIR spectrum showed that the shoulder at 1458.22 cm^{-1} and 1628.14 cm^{-1} are present in the spectrum evidence of covalent bond between nitrogen and oxygen tremble and the closely spaced bands 625.36 cm^{-1} are presents in the spectrum evidence of AgZnONCs tensional tremble, respectively (Fig. 1). Also, the broad band at 3426.47 cm^{-1} was allocated of hydration water [18]. The XRD patterns of AgZnONCs (Fig. 2) was associated with standard information of International Centre of Diffraction Data (ICDD). The average crystallite size of the nano-crystals were designed using the Debye-Scherer Equation. The average crystallite size of the AgZnONCs were about 12 nm. Estimated size of nano-crystals, as well clustering and spherical morphology was showed via SEM image (Fig. 3).

3.2 | Anti-tubercular activity of AgZnONCs and AgZnONCs+rifampicin

Consistent to the results (Tables 4 and 5), none of the dilutions of AgZnONCs were not able to kill of 10^{-1} (15×10^7 CFU ml^{-1}) *H_{37}RvMtb*. Statistical data also did not show any significant difference between control group and other dilutions (p-value >0.05). However, different behavior of

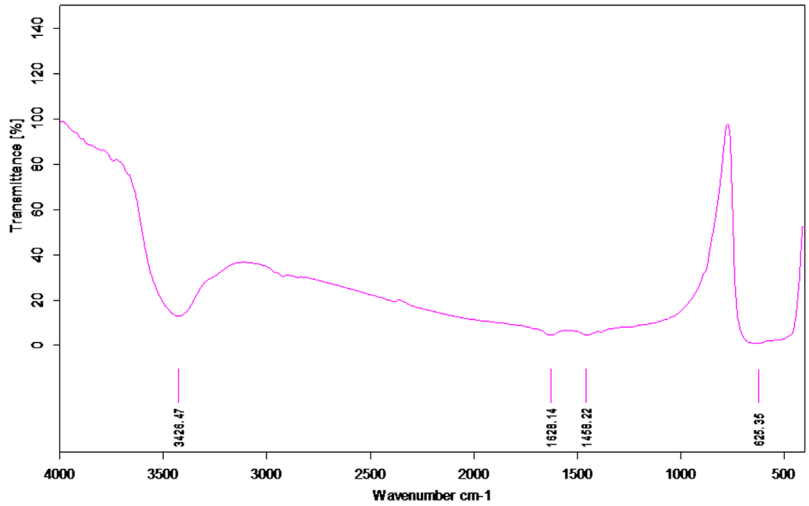

FIGURE 1 FT-IR spectra of AgZnONCs. The spaced band 625.36 cm^{-1} is presents in the spectrum evidence of AgZnONCs tensional tremble

$H_{37}RvMtb$ in dilution of 10^{-2} (75×10^6 CFU ml^{-1}), 10^{-3} (37.5×10^5 CFU ml^{-1}), and 10^{-4} (18.75×10^4 CFU ml^{-1}) were observed (Table 4). Statistical analysis showed significant differences between the CFU of *$H_{37}RvMtb$* at dilution of 4096 µg ml^{-1} and control group in all dilutions (p-value <0.05) (Fig. 4). Results showed that, mixed AgZnONCs+Rifampicin could kill about 10^{-1} (15×10^7 CFU ml^{-1}) *$H_{37}RvMtb$* in dilution of 1024:2.5 µg ml^{-1}. Also, it could kill about 10^{-2} (75×10^6 CFU ml^{-1}), 10^{-3} (37.5×10^5 CFU ml^{-1}), and 10^{-4} (18.7×10^4 CFU ml^{-1}) *$H_{37}RvMtb$* at ratio of 1:32 (MBC = 512:1.25 µg ml^{-1}). In comparison with AgZnONCs, the MBC of AgZnONCs +Rifampicin in dilution of 10^{-2}, 10^{-3}, and 10^{-4} was reduced until eight times (Table 5). Statistical analysis presented significant differences in dilution of 1:32, 1:16 (p-value <0.05) (Fig. 5).

3.3 | MTT assay

According to MTT assay (Fig. 6), 4096 µg ml^{-1} of AgZnONPs did not have any cytotoxicity effect on THP-1 cell line. In fact, the number of viable THP-1 cells was increased with reducing of the concentration of AgZnONPs. Also, dilutions of 512 µg ml^{-1} and 256 µg ml^{-1} the viability percent of THP-1 cells reached to more than 80%, compared with the control group (Fig. 6).

3.4 | The infectious THP-1 cell lines by *$H_{37}RvMtb$* and treated with AgZnONCs and AgZnONCs+rifampicin

The results of culturing infectious THP-1 cells with 10^{-2}, and 10^{-4} McFarland of *$H_{37}RvMtb$* and treated with different dilution of AgZnONCs, compared to the control group

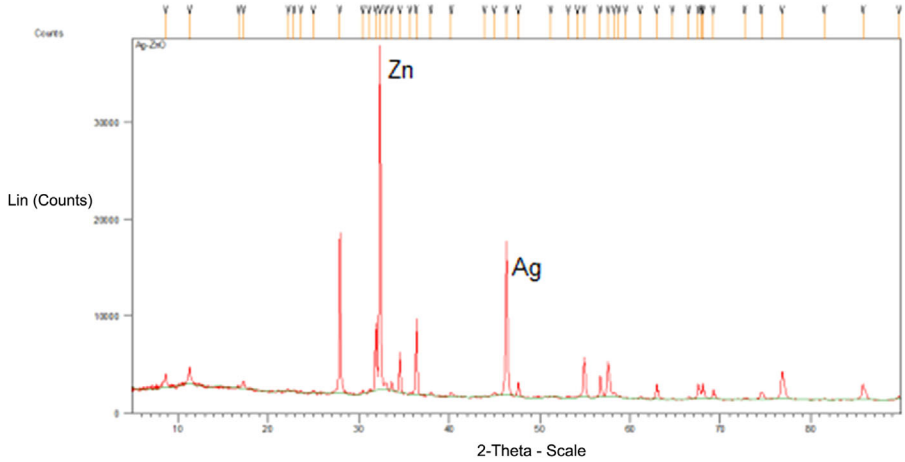

FIGURE 2 XRD pattern of AgZnONCs displays a typical specrtum of the nano crystalline Ag and ZnO prepared with oxalate decomposition route. These results reveal that although the ZnO peaks are more evident, two phases (Ag and ZnO) co-exist

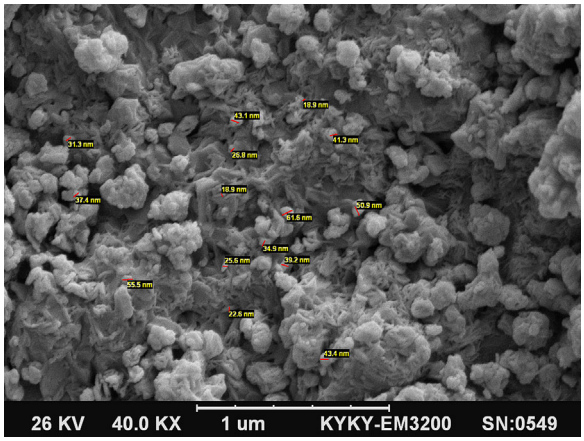

FIGURE 3 SEM image of AgZnONCs showed that metallic particles were exactly in the shape of spherical and cluster

defectively. Meanwhile, it was found that further dilutions do not have any anti bactericidal effects on phagocytic bacteria. While, anti-tubercular behavior of AgZnONCs+Rifampicin was intensified in present of 18.75×10^4 CFU ml^{-1} of *Mtb*. So, it can be strongly believe that 8192:20 μg ml^{-1} till 512:1.25 μg ml^{-1} dilution of AgZnONCs+Rifampicin able to completely kill all of bacteria into the macrophages. Also, the weak growth of $H_{37}RvMtb$ colonies was shown in 256:0.625 μg ml^{-1} (Fig. 8).

4 | DISCUSSION

TB treatment has become a major problem due to some factors related to bacterial infection. First, the body's defense mechanism in tuberculosis patients are incompetent. For this reason, pulmonary tuberculosis occurs only in a few number of people that infected with tuberculosis. In addition, pathological anatomy of lesions with high leukocyte infiltration and their central calcification that make difficult the penetration of drug at appropriate concentrations. Also, *M. tuberculosis* has some unfavorable characteristics. For instance, their metabolism is slow and some of them take place within cells and may remain a long time in calcified and fibrous lesions. These factors along with the fact that most anti-TB drugs only have bacteriostatic activity prolong the treatment of this disease. Other problems of Tb treatment are

TABLE 4 The MBC outcomes of AgZnONCs were compared to the different dilutions of $H_{37}RvMtb$ (*p-value <0.05 are representative of significant values and **p-value >0.05 are indicative of non-significant values)

Ratios and concentration (ppm) of AgZnONCs										
Dilution of *H37Rv* (CFU ml^{-1})	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Control
	8192	4096	2048	1024	512	256	128	64	32	
10^{-1} (15×10^7)	+2**	+3**	+3**	+3**	+3**	+3**	+3**	+3**	+3**	+4
10^{-2} (75×10^6)	0*	MBC*	+1**	+2**	+2**	+2**	+2**	+3**	+3**	+4
10^{-3} (37.5×10^5)	0*	MBC*	+1**	+2**	+2**	+2**	+3**	+3**	+3**	+4
10^{-4} (18.75×10^4)	0*	MBC*	+1**	+1**	+1**	+2**	+3**	+3**	+3**	+4

showed that 8192 μg ml^{-1} of AgZnONCs able to kill of 10^{-2} McFarland of $H_{37}RvMtb$ into the THP-1 cells, so weakly. However, it has been notice that it cannot be active not only by 4096 μg ml^{-1}, but also in other dilutions. In low concentration of bacteria, no colonies were observed in the 8192 μg ml^{-1}. However, it had a weak growth. Also, it was found that the CFU increases after another in 2048 μg ml^{-1} to 32 μg ml^{-1} dilutions, gradually (Fig. 7).

By the way, it has been demonstrated that AgZnONCs +Rifampicin is able to kill 10^{-2} $H_{37}RvMtb$ into the THP-1 cell lines in dilution of 8192:20 μg ml^{-1}, 4096:10 μg ml^{-1}, 2048:5 μg ml^{-1}, completely and in dilution of 1024:2.5 μg ml^{-1},

TABLE 5 The MBC results of AgZnONCs+Rifampicin were compared to the different dilutions of $H_{37}RvMtb$ (*p-value <0.05 are representative of significant values and **p-value >0.05 are indicative of non-significant values)

Ratios and concentration (ppm) of AgZnONCs+rifampicin										
Dilution of *H37Rv* (CFU ml^{-1})	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Control
	8192:20	4096:10	2048:5	1024:2.5	512:1.25	256:0.625	128:0.312	64:0.156	32:0.078	
10^{-1} (15×10^7)	0*	0*	0*	MBC*	+1**	+1**	+3**	+3**	+3**	+4
10^{-2} (75×10^6)	0*	0*	0*	0*	MBC*	+1**	+2**	+3**	+3**	+4
10^{-3} (37.5×10^5)	0*	0*	0*	0*	MBC*	+1**	+2**	+3**	+3**	+4
10^{-4} (18.75×10^4)	0*	0*	0*	0*	MBC*	+1**	+2**	+3**	+3**	+4

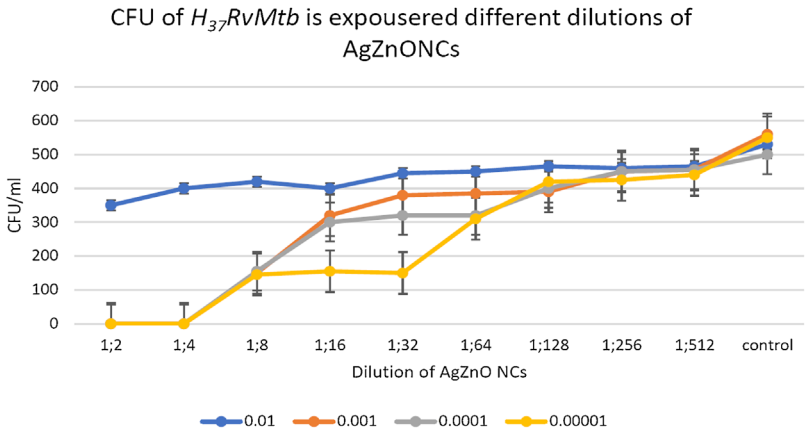

FIGURE 4 Anti bactericidal effects of different ratios of AgZnONCs in present of 1:10 (10^{-1}), 1:100 (10^{-2}), 1:1000 (10^{-3}), and 1:10,000 (10^{-4}) McFarland of *$H_{37}RvMtb$*

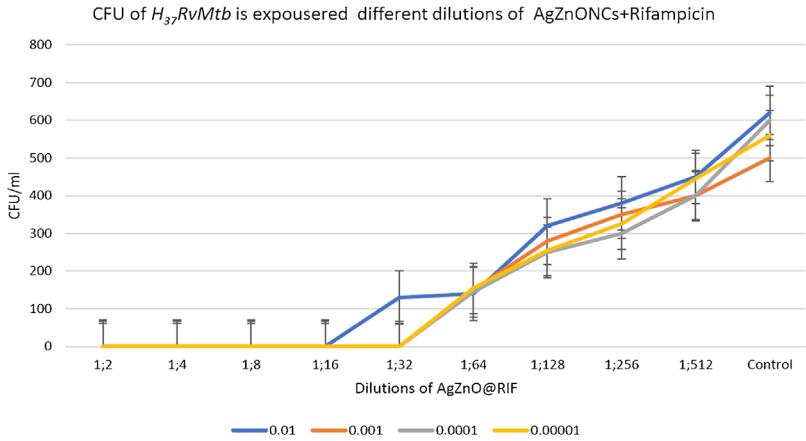

FIGURE 5 Anti bactericidal effects of different ratios of AgZnONCs+rifampicin in present of 1:10 (10^{-1}), 1:100 (10^{-2}), 1:1000 (10^{-3}), and 1:10,000 (10^{-4}) McFarland of *$H_{37}RvMtb$*

the emergence of antibiotic resistant *M. tuberculosis*, and cytotoxic effects of new antibiotics [19]. According to the previous study, silver (AgNCs) and zinc oxide nano-crystals (ZnONCs) have antibacterial effects on wide ranges of bacteria, also have synergistic effects on each other [16,20]. Recently, it was found that ZnO colloidal nanoparticles are not toxic against human lungs normal fibroblasts (MRC-5) and human macrophage (THP-1) cell lines [12]. Despite of

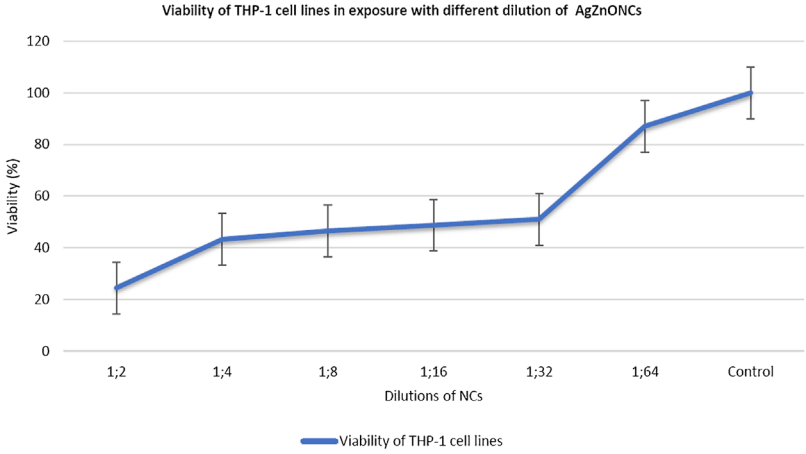

FIGURE 6 Viability of THP-1 cell lines in exposure with different dilution of AgZnONCs

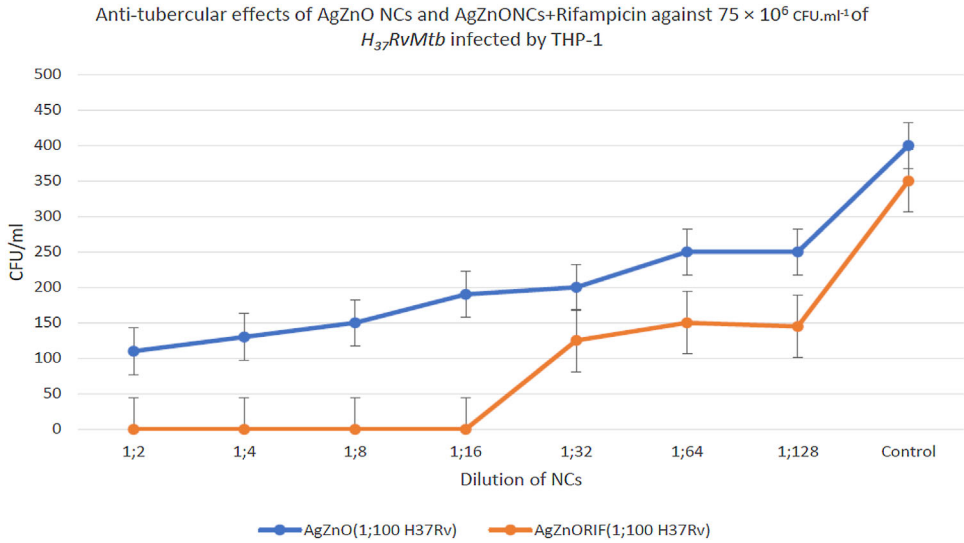

FIGURE 7 Comparison between antibacterial effects of AgZnONCs and AgZnONCs+rifampicin against 75×10^6 CFU ml^{-1} (1:00 McFarland) of *H_{37}RvMtb* infected by THP-1 in the presence of different dilution of AgZnONCs

this fact that ZnONCs revealed very strong activity against gram positive and gram negative bacteria.

According to Jafari et al. [21], mixed silver and zinc oxide nano colloidal particles had anti-tubercular impact on H_{37}Rv*Mtb*. They also fined that 40 ppm of rifampicin could not be able to eliminate 75×10^4 CFU ml^{-1} *H_{37}RvMtb* after phagocytized by THP-1 cell lines. According to current study, combination of 1024 μg ml^{-1} of AgZnONCs with 2.5 ppm of rifampicin was able to eliminate 75×10^4 CFU ml^{-1} H_{37}Rv strain of *MTB* after phagocytized. While, AgZnONCs could not be able kill all bacteria into the THP-1 cells. Sarkar et al. [22] investigated on cytotoxicity of silver colloidal NPs in various aspects with THP-1 as well as the innate immune response in the face of *H_{37}RvMtb*. The results of this study showed that the viability of THP-1 cells reduced in the present of AgNCs. López-Heras et al. [23] had talked about the antibacterial properties of AgNCs and suggested that AgNCs can be used to inhibit the growth of bacteria in respiratory infections. In this study, they investigated antibacterial properties of AgNCs that were uptake the *Mtb* infected with macrophages. Similar to current research, they infectious THP-1 cells with *Mtb* and investigated various cellular structures and their changes [23]. Recently, using of conjugated nano-medicine in order treatment of TB has

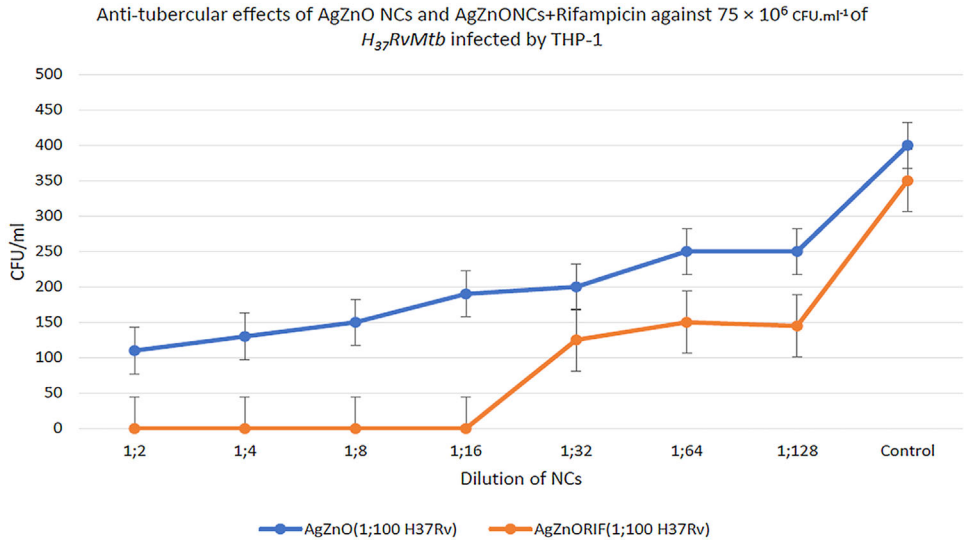

FIGURE 8 Comparison between antibacterial effects of AgZnONCs and AgZnONCs+rifampicin against 18.5×10^6 CFU ml^{-1} (1:10,000 McFarland) of *H_{37}RvMtb* infected by THP-1 in the presence of different dilution of AgZnONCs

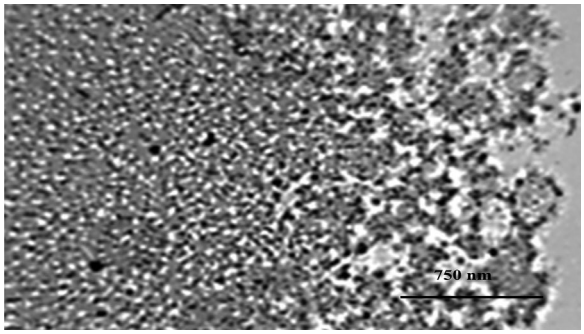

FIGURE 9 TEM image of the THP-1 cell treated with AgZnONCs with magnification of 750 nm. Clearly, it is obvious that NCs have penetrated into the macrophage cell line

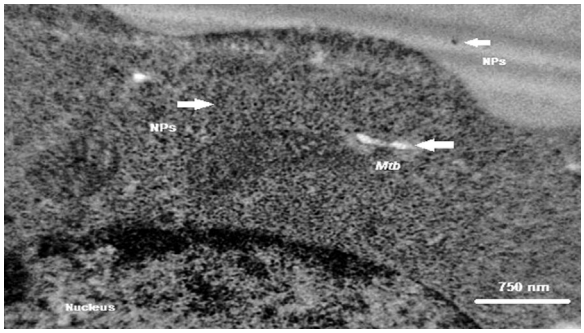

FIGURE 10 TEM image show that some phagocytosis $H_{37}RvMtb$ and nano-crystals are afloat into the THP-1 cell cytosol

been changed to the title which is interesting for researchers. Leidinger et al. [24] considered the antibacterial effects of isoniazid-filled Fe_2O_3 as a nano-medicine in the treatment of TB against phagocytic *Mtb*. Their results indicated that isoniazid-filled Fe_2O_3 had a very strong antibacterial activity against *Mtb* in the *in vivo* and *ex vivo*. They introduced isoniazid-filled Fe_2O_3 as "Trojan horses." Mohanety et al. [25] investigated the antibacterial peptides and the AgNPs against a variety strains of *Mtb*. AgNPs were synthesized with a biological method via bacteria, fungi, and plants and were combined with various antibacterial peptides. Hala achieved some results on the antibacterial effects of Au Nano-rods (AuNRs) and AuNRs+Rifampin against *Mtb* and phagocytic *Mtb* with macrophage cell lines [26]. According current results, the AgZnONCs able to destroy all the *Mtb* in *in-vitro* condition. In fact, after phagositosis both of AuNRs and AgZnONCs had penetrated into the macrophage. Interestingly, they figured out that AuNRs+Rifampin able to remove all of bacteria into macrophage. But AgZnONCs was able to kill about 10^{-2} (75×10^6 CFU ml^{-1}) *Mtb* into macrophage at dilution of 1:16 (1024 μg ml^{-1}), without the involvement of the rifampicin. It was confirmed the presents of nano-crystals into the macrophage via TEM images (Fig. 9). Also, TEM image showed *Mtb* into the cytosol of macrophage (Fig. 10) and the MTT assay showed that more than 50% of the cells were alive in concentration of 4096 μg ml^{-1}. However, there were no reliable and the concentration seemed somehow toxic. Conversely, it was demonstrated synergistic effects between the AgZnONCs and the rifampicin. In fact, it was indicated that AgZnONCs+Rifampicin is able to kill about 10^{-4} bacteria at dilution of 1:32. The fact is that, more than 80% of macrophage cells which were exposed to AgZnONCs were alive and it was a good news. TEM image showed that AgZnONCs were not able to kill the bacteria in *ex-vivo* condition, completely (Fig. 10). Also, Figs. 11 and 12 were designed as a schematic illustration of anti-tubercular impact of AgZnONCs against $H_{37}RvMtb$. This study also showed that antibacterial properties of AgNPs and ZnONPs could be increase by mixing nanoparticles with antibiotics such as rifampicin, which has more for permeating into lung tissue fibrin layer. Studies have shown that some magnetic NPs are able to induce capable macrophage for phagocytosis. However, it can be pointed as spark for future researches for synthesis of mixed metal oxide and magnetic NPs with

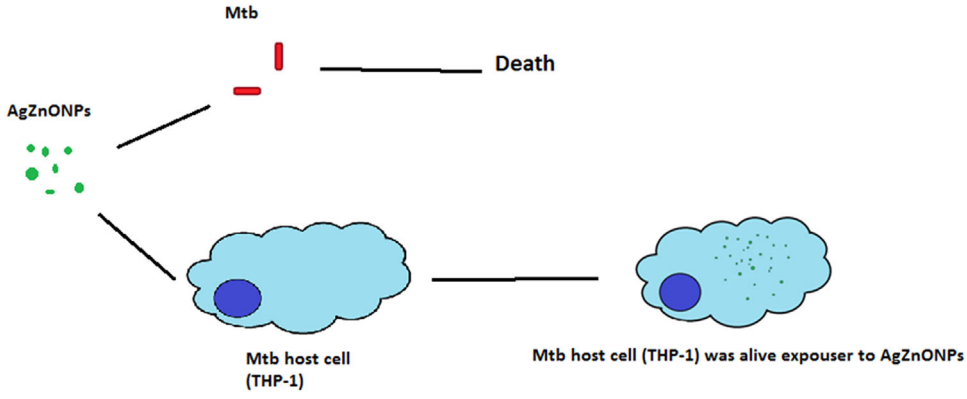

FIGURE 11 Schematic illustration of anti-bacterial effects of AgZnONCs against $H_{37}RvMtb$ (*in vitro*) and viability of THP-1 cells as a host cell of $H_{37}RvMtb$ in present of different dilution of AgZnONCs

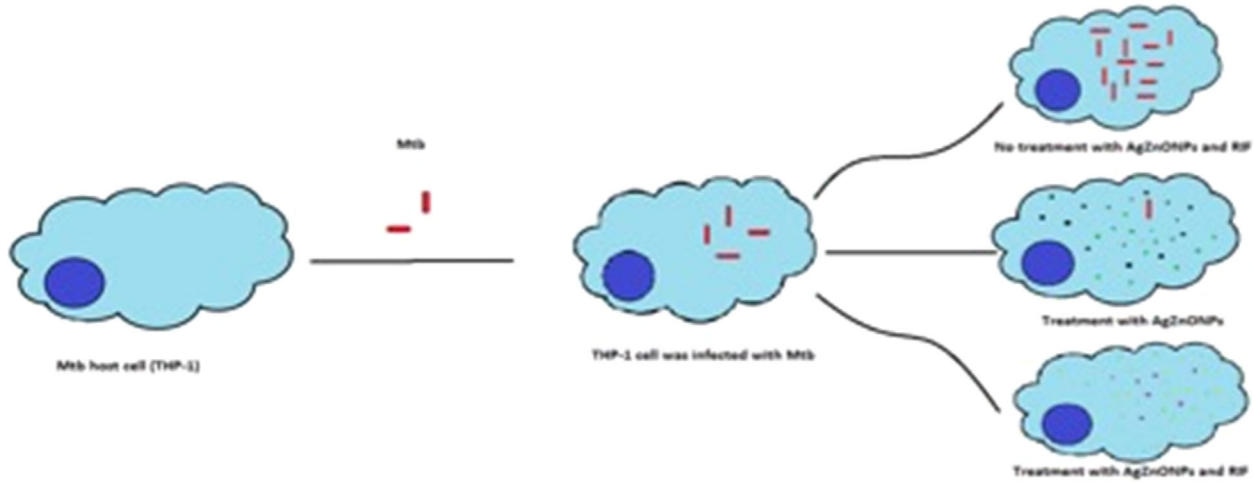

FIGURE 12 The THP-1 cell infected with *H_{37}RvMtb* and treated with AgZnONCs, as well as AgZnONCs+rifampicin compared to the control group. All of the phagocytic bacteria were eliminated in present of AgZnONCs+rifampicin

antibacterial as well macrophage inducer properties against inner cellular bacteria especially *Mtb*.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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